RESEARCH ARTICLE

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Evaluation of HPV DNA positivity in colorectal cancer patients in Kerman, Southeast Iran

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Abstract

Background: The HPV virus is known to be oncogenic and associations with many cancers has been proven. Although many studies have been conducted on the possible relationship with colorectal cancer (CRC), a definitive role of the virus has yet to be identified. **Method:** In this cross-sectional study, the frequency of HPV positivity in CRC samples in Kerman was assessed in 84 cases with a mean age of 47.7 ± 12.5 years over two years. Qualitative real time PCR was performed using general primers for the L1 region of HPV DNA. **Results:** Out of 84 CRC samples, 19 (22.6%), proved positive for HPV DNA. Genotyping of positive samples showed all of these to be of high risk HPV type. Prevalence of HPV infection appears to depend geographic region, life style, diet and other factors. **Conclusion:** In our location frequency of CRC is low, and this limited the sample size for evaluation of HPV DNA. The most prevalent types were HPV types 51 and 56. While HPV infection may play an important role in colorectal carcinogenesis, this needs to be assessed in future studies.

Keywords: Colorectal cancer- HPV- real time PCR- HPV genotype

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Introduction

Colorectal cancer (CRC), also called colon cancer, includes cancerous growth of the cells in the colon, sigmoid and rectum. The CRC is the fourth most common

Cancer in men and second in women, according to the Ministry of Health and Medical Education of IRAN (Bouvier and Launoy, 2015; Dolatkhah et al., 2015). It is the third most common cancer in the world with an estimated 1,200,000 new cases per year, of which 608,000 are causing death (Aran et al., 2016). In Iran colorectal cancers, 61.83 % were colon cancer, 27.54 % rectal cancer, 7.46 % recto sigmoid cancer, and 3.10% anal cancers (Rafiemanesh et al., 2016). Colon cancer is the fourth cause of death from cancers in the world. The number of new cases has risen steadily since 1975 onwards. Across the world, cancer accounts for 10.1% of all cancers in women and 9.4% in men (Marley and Nan, 2016). The risk of developing colorectal cancer is affected by many factors such as gender, age, alcohol consumption, fiber deficiency or high fat diet, hereditary conditions, family history of colorectal cancer, individual history of polyp colon, inflammatory bowel disease, and factors Varied genetic (Pahlavan and Kanthan, 2006). According to some hereditary studies, about 20% of patients diagnosed with this type of cancer have a genetic background, and the presence of at least 2 patients with colorectal cancer in a family has a 2 to 3-fold increase in outbreak risk. While some other studies have shown that 75 to 95 percent of colon cancer cases were found in people who were not hereditary (Matuchansky, 2017).Another risk factor for colorectal cancer is viral infections. Viral etiology in human cancers is an interesting topic, and so far a small number of viral species have been identified as human timorous viruses, including EBV, hepatitis C (HCV), hepatitis B (HBV) , papillomavirus (HPV) and human herpes virus 8 (HHV8) (Aran et al., 2016; Leila et al., 2016; Al-Azri et al., 2017). In the last studies, researchers have examined the relationship between HPV and colorectal cancer. More than 100 different types of HPV have been isolated from various human samples which can cause benign (wart) and malignant tumors (cervical cancer). HPV types 16, 18, 54, 56, 58, 31 have been classified to High risk groups.HPV types 6 ,11 are low risk viruses that cause wart , benign and subclinical disease (Afshar et al., 2013b). Colorectal cancer begins with epithelial cells and anal tissues, where HPV is known to be associated with malignancies. Some of studies showed 14-84% of the cases of colorectal cancer positive for the presence of the DNA of the papillomavirus, and others reported a small or no presence of DNA of the papillomavirus (Baandrup et al., 2014; Bai et al., 2017) .Therefore, the relationship between HPV infection and colorectal cancer is not clear. The HPV carcinogenic potential is due to the ability to

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penetrate and integrate into the host DNA, to be present in the cell cycle and to stop the function of the proteins P53 and RB (Retinoblastoma) (Motoyama et al., 2004). Infection with different types of human papillomavirus is a necessary but not sufficient condition for the formation of neoplastic processes of various organs after a long time of infection with HPV(Yahyapour et al., 2012) .The known carcinogenicity of E6 and E7 human papillomavirus is interfering with cell cycle and stimulation a few molecular processes lead to the malignancy of the infected cell by the virus (Chen et al., 2012).HPV acts as a risk factor for colorectal cancer, preventive measures such as vaccination and the use of antiviral drugs appropriate for the health plan of the infected communities (Li et al., 2015; Vorno et al., 2017). Therefore, in this study, we have detected HPV DNA in colorectal cancer by Real Time PCR, whereas others methods have been unable or low sensitive to demonstrate the presence of HPV in CRC. As due to the high prevalence of colorectal cancer, especially in the elderly, and the power of invasion and metastasis, and viral infection in cancer etiology is concerned, the relation of HPV with CRC carcinogenesis can have a significant impact on Patient care, we concluded that a study aimed at determining the prevalence of HPV in patients with colorectal cancer using PCR technique.

Materials and Methods

Sample collection

A total of 90 samples of paraffin blocks of colorectal cancer were collected. However, due to the unavailability of the pathological records of 6 patients, 84 samples were included in the study during June 2012 to September 2015, in the pathology department of Afzalipour Hospital in Kerman. Paraffin embedded blocks were processed using xylen for remove of paraffin that possibility of isolating DNA for HPV detection assays. This method has received approval for clinical use from the U.S. Food and Drug Administration.

Deparaffination samples

Paraffinated blocks from the 84 tumor samples were cut in 3-µm sections and 5 sections, patients were collected in the same micro-centrifuge tube. Samples were de-waxed in 800 µl xylen; all micro-centrifuge tube located for 10 min in a 60 °C heated block and centrifuged at 10,000 rpm for 1 minute, supernatant was removed. This step was then repeated three times. Add 500 µl absolute ethanol, centrifuge at 10,000 rpm for 1 minute, the samples were then dried in a 70°C heated block with open lids for 5-10 min for remove residual ethanol (Reza et al., 2012).

Tissue digestion

According to samples (biopsy or Paraffinated blocks), 200-400 µl of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 mMTris, 20 mMNaCl, 200 mM EDTA; PH=7.4 (25°C)]. To all tubes added 20-40 µl proteinase K, Samples were gently vortexes and located for 10 min in a 60°C heated block, and all samples were subsequently incubated at 37°C overnight (Reza et al., 2015).

DNA Extraction

The next day, 200 µl of Binding Buffer [6 M Guanidine- Hcl, 10mM Urea, 10mM Tris-Hcl, 20% Tritonx-100 (v/v); PH=4.4 (25°C)] was added to each tube with gently vortex. DNA was isolated using a QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA pellets were suspended in 70µl of pre-warmed Elution buffer and stored at -70°C until use.

Qualitative Real Time PCR

After DNA extraction, for detection and screening positive samples with HPV, a qualitative Real time PCR based on SYBR Green was done. The primers used in this study were general primers from L1 region of HPV : MY09 and MY011 pairs (MY09: 5'-CGT CCM AAR GGA WAC TGA TC-3' and MY011: 5'-GCM CAG GGW CAT AAY AAT GG-3') (Yahyapour et al., 2012).

INNO-LiPA HPV detection and genotyping

After performing a PCR test and identifying positive samples, the Inno-lippa test was performed to identify the types of HPV. The INNO-LiPA® HPV Genotyping Extra II (Fujirebio Diagnostics, Sweden) kit was used for this experiment, the steps of which have been described below (Afshar et al., 2013a).

A) PCR amplification of HPV DNA

Broad-spectrum HPV DNA amplification was performed using a short PCR fragment assay (INNO-LiPA HPV detection/genotyping assay, Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame and allows detection of at least 43 different HPV types. The SPF10 PCR was performed with a final reaction volume of 50 µl containing 10 µl of the isolated DNA sample, 10 mmol/liter Tris-HCl (pH 9.0), 50 mmol/ liter KCl, 2.0 mmol/liter $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 µmol/liter of each deoxynucleosidetriphosphate, 15 pmol each of the forward and reverse primers tagged with biotin at the 5'end, and 1.5 U of AmpliTaq Gold (Perkin-Elmer). The mixture was incubated for 9 min at 94°C, 40 cycles of 45s at 45°C, and 40 cycles of 45s at 72°C, with a final extension of 5 min at 72°C. Each experiment was performed with separate positive and negative PCR controls. The presence of HPV DNA was determined by hybridization of SPF10 amplimers to a mixture of general HPV probes recognizing a broad range of HPV genotypes, in a micro titer plate format, as described previously (Ghasemian et al., 2013; Eftekhaar et al., 2017).

B) HPV genotyping by reverse hybridization using the INNO-LiPA HPV genotyping system

A poly (dT) tail was enzymatically added to the 3' end of each of 25 oligonucleotides specific for 25 different types, namely, types 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. The tailed probes were applied as horizontal lines to membrane strips (manufactured by Labo Biomedical Products bv, Rijswijk, The Netherlands). The HPV genotyping assay was performed as described previously.

Briefly, equal volumes (10 µl each) of the biotinylated PCR products and denaturation solution (400 mmol/ liter NaOH, 10 mmol/liter EDTA) were mixed in test troughs and incubated at room temperature for 5 min, after which 1 ml of prewarmed (37°C) hybridization solution was added, followed by the addition of one strip per trough. Hybridization was performed for 1 h at 50±0.5°C in a closed water bath with back-and-forth shaking. The strips were then washed twice with 1 ml of wash solution at room temperature for 20 s and once at 50°C for 30 min. Following this stringent washing, strips were rinsed twice with 1 ml of a standard rinse solution. Strips were then incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution, at 20 to 25°C for 30 min, after which strips were washed twice with 1 ml of rinse solution and once with standard substrate buffer; color development was initiated by the addition of 5-bromo-4- chloro-3-indolylphosphate and Nitrobluetetrazolium to 1 ml of substrate buffer. After 30 min of incubation at room temperature, the color reaction was stopped by aspiration of the substrate buffer and addition of distilled water. After drying, the strips were visually interpreted using a grid (Yahyapour et al., 2012; Karbalaie Niya et al., 2017).

Statistical analysis

Chi square and Fisher's exact Tests were used to analyze the data obtained by SPSS 11.5 software (SPSS Inc, Chicago; USA). The differences or association with $p<0.05$ were considered statistically significant.

Results

A total of 84 paraffin tissue samples of colectomy specimens were selected from men and women with colorectal cancer diagnosed between 2012 and 2014. All biopsy specimens were diagnosed with colorectal cancer by two pathologists from the pathology department of Kerman hospitals. All required information, including age and sex, was extracted from the records of patients archived in the laboratory. The mean age of the studied patients with colorectal cancer at the time of diagnosis was 47.7 ± 12.5 with a range of 18 to 85 years old, of which 51 were male(60.7%) and 33 (39.3%) were female

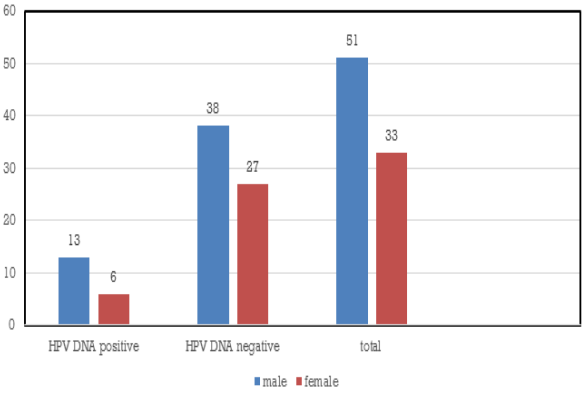

Figure 1. Frequency of HPV DNA in Different Sex of Colorectal Cancer

Table 1. Distribution Sex and Age Group of Patients in This Study

Age group	Male (%)	Female (%)	Total (%)
< 30	3 (60%)	2 (40%)	5 (6.1%)
40 – 31	7 (56.25%)	9 (43.75%)	16 (19.04%)
50 – 41	7 (70%)	3 (30%)	10 (11.90%)
60–51	14 (82.35%)	3 (17.64%)	17 (20.23%)
> 60	20 (55.55%)	16 (44.44%)	36 (42.85%)
Total	51(60.7%)	33 (39.28%)	84 (100%)

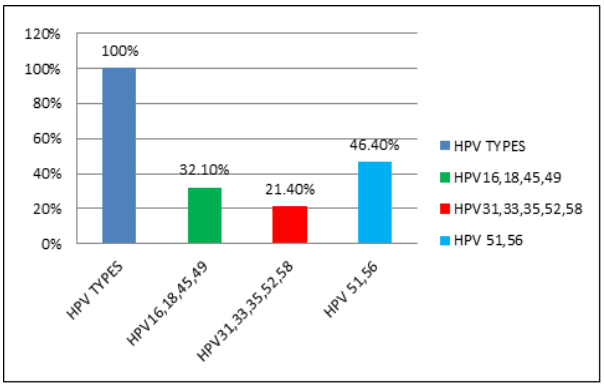

Figure 2. Frequency Different Types of HPV in Total Samples

(Table1). Of the 84 cancer cases, according to the results, 22.6% (19 samples) had the genome of the papillomavirus, which included different high risk HPV types (Figure1). The highest frequency was for HPV types 51 and 56, and the lowest was for the HPV types 31, 33. However, the prevalence of HPV in men was higher than women, but there was no significant difference between there (Figure 2) .As shown in Figure 3, colorectal tumors are divided into three grades: Poor, Moderate, and Well. The highest frequency was found in Moderate (74.99%), with an abundance of HPV types 51, 56 (33.33%), HPV types 16, 31, 33 (25%) and HPV types 18, 39.45 (16.66%). Figure 4 shows the frequency of HPV in both invasive and noninvasive states. As you can show, the frequency of HPV in vascular invasion is more than the absence of invasion (26.3%). The highest prevalence in the vascular invasion group is related to HPV types 51 and 56, 58.33%, HPV types 18, 39, 45 at 16.6%, HPV types 16, 31, 33 at

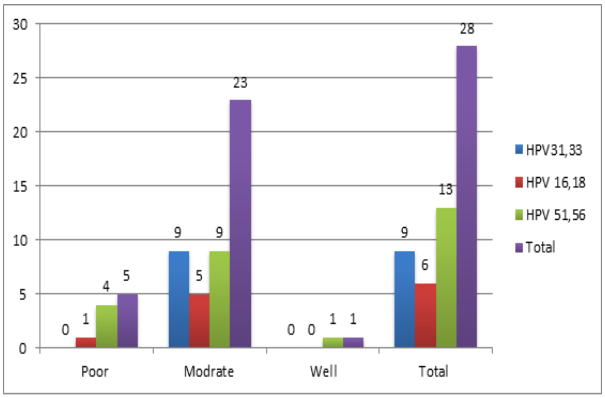

Figure 3. Distribution Frequency of HPV Types in Different Grades of CRC

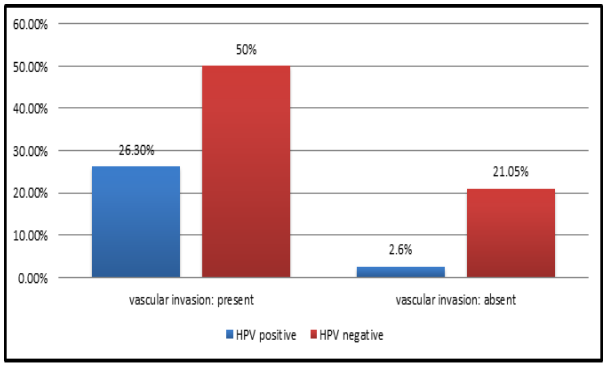

Figure 4. Frequency of HPV DNA in Two Types of Vascular Invasion

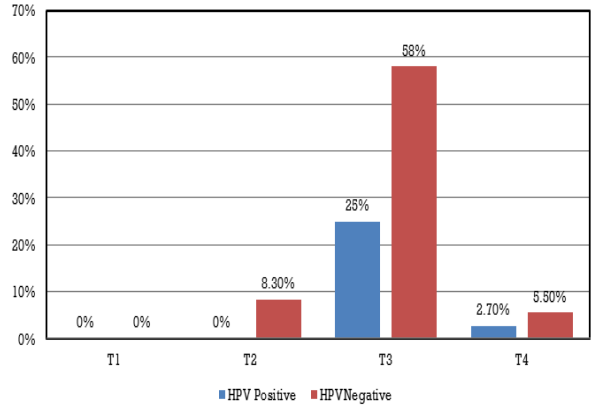

Figure 5. Frequency of HPV DNA in Different Tpes of Local Extension

6.66%. In the group that had no vascular invasion, only HPV types 16 and 31 were positive at 8.3%.In figure 5 Frequency of HPV DNA in different types of Local Extension was shown and in figure 6 Frequency of HPV DNA in prineural invasion and Lymph node metastasis . There were no significant relation between types of Local Extension and prineural invasion and Lymph node metastasis with HPV DNA (P.Value >0.05).

Discussion

Recent studies have shown that 15% of the existing cancers are due to the infection of the oncogenic viruses, which presence of papillomavirus is commonly found in human tumors, as it can be an etiologic factor of cancers (Aran et al., 2016) . Also, the presence of HPV DNA in the colorectal cancer, as one of the most common cancers in developed countries, shows that it's potential as a carcinogenic factor in this cancer (Baandrup et al., 2014). Therefore, due to the high prevalence of colorectal cancer especially in the elderly population (over 50 years old age) and the power of the invasion of this cancer, we decided evaluate frequency of HPV and its corresponding types in patients with colorectal cancer using the Real-Time PCR technique. In this study, frequency of HPV DNA was 6.22% (19 samples) for colorectal cancer cases. The highest frequency was related to the types 51 and 56 (15.5%) and the lowest frequency was related to the HPV types 31 and 33 (7.1%), however, the frequency of HPV types 16, 18,58 types was 10.1% .In this study mix

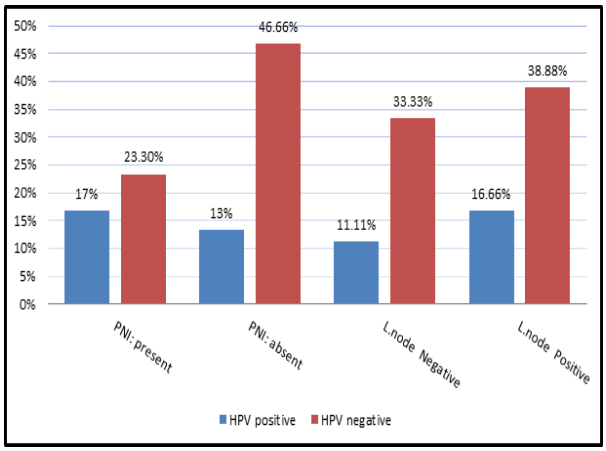

Figure 6. Frequency of HPV DNA in Prineural Invasion and Lymph Node Metastasis

typing of HPV infection were seen (Table 2). A study by Bernabe-Dones in 2016 showed that the prevalence of DNA-HPV in colorectal cancer cases was 42.2%, which was detected by PCR and HPV-16 was detected in 63.2% of the HPV-positive colorectal tumors. This was much higher than our study, which may be due to the use of more samples (Bernabe-Dones et al., 2016). On the other hand, our study limitation was based on the use of old paraffin specimens, which decrease of DNA and damage it, so; false negative results. Distribution and frequency of HPV depends on the geographical area and demographic factors (Christensen et al., 2017). A further study by Argentina's Pérez in 2005 and 2010 found that the prevalence of DNA-HPV in colorectal cancer cases was 74% and 44%, respectively. In these studies, the PCR method was utilized using universal primers that naturally increased the frequency .The geographic distribution of the study in two countries can be explained by the reason for this difference (Perez et al., 2005; Perez et al., 2010). In a study by Motlagh in Iran, frequency of DNA-HPV was similar to our study, as the frequency of type 18 was higher than that of type 16 (Motlagh et al., 2007). The result of the Liu study was similar to that of Perez in 2010, which was the HPV 16 most frequent type, and a US study in 2016 showed that 65% of the HPV types were between the two types 16 and 53 (Perez et al., 2010; Liu et al., 2011). In a Meta analysis study by Damin in 2013, the HPV prevalence was 31.9% (95% CI: 19.3-47.9). It was lowest in Europe (14.1%, 95% CI: 4.9-34.1) and highest in South America (60.8%, 95% CI: 42.7-76.4). Eight studies showed the results of HPV typing in 302 HPV-positive colorectal carcinomas. HPV 18 was more frequently found in colorectal cancer cases from Asia (73.34%, 95% CI: 44.9-90.7) and Europe (47.3%, 95% CI: 34.5-60.4). In contrast, HPV 16 was more prevalent in colorectal tumors from South America (58.3%, 95% CI: 45.5-69.9). The analysis of five case-control studies reported an increase in colorectal carcinoma risk with HPV positivity (OR = 10.04; 95% CI: 3.7-27.5), So, The results show that an evidence for association between HPV infection and colorectal cancer (Damin et al., 2007). Also, in the United States, the incidence of HPV was higher in people over 55 years of age, although there was no significant difference. The Bernabe-Dones study

in United States showed that although the prevalence of HPV was higher in women than in men, the difference was not significant (Bernabe-Dones et al., 2016). Therefore, according to these studies, the gender is not significantly associated with HPV due to the type of race, gender, and cultural differences existing in countries and the difference in the HPV induced colorectal cancer in both males and females may not be established. Although the prevalence of HPV types in men was higher than that of women, this difference was not significant between two age groups. The prevalence of HPV in colorectal cancer is variety in different parts of the world, indicating its dependence on culture, geographical location, religion, and diet; such as, in Poland the frequency of HPV-DNA types 16 and 18 was 67%, also in 56% and 28% of normal polyps and mucus HPV DNA was detected (Dahms and Nowicki, 2015) .In Turkey HPV DNA was detected in 32% of normal tissues and 81.2% of cancerous tissue. In this study, only types 16, 18, 6, 11 and 33 were detected (Aykan et al., 2015). In Saudi Arabia HPV-DNA was detected in only 4 samples of the whole sample (Gazzaz et al., 2016). In Argentina HPV-DNA was detected in 33 (44%) cases of all specimens. In China prevalence of HPV in colorectal cancer was 37.5% and finally in Tehran 35% of the cases were positive for HPV-DNA, with HPV18 at 32% Cases and HPV16 were observed in 18% of cases (Perez et al., 2005; Liu et al., 2011; Chen et al., 2012). Base on results of our study, the prevalence of HPV types in the age group was greater than 50 than those older than 50 years of age, although there was no significant difference between the two age groups. Since various factors such as age and family history play a role in increasing the risk of colon cancer, people with aging due to genetic changes may also progress to cellular changes and become involved with the oncogenic viral infection of the cancer and an increase in the incidence of HPV in patients older than 50 was found. The highest frequency was found in moderate grade, 63.8% and the lowest was found in well grade 0%. Also, about 50% of tumors with vascular invasion were positive for HPV, but there was no significant relationship between vascular invasion and HPV. In the present study, the prevalence of HPV in men was almost twice as high as women, but there was no significant correlation between sex and HPV in CRC (P.Value =0.14). The distribution of HPV in the local extension and T3 type was 25%. The frequency of HPV in tumors that had prineural invasion and metastasis to lymph nodes was 17% and 16.6% respectively. There was no significant relationship between prineural invasion and metastasis to lymph nodes with HPV. According to our results, 6.22% (19 samples) had the genome of the papillomavirus; the highest frequency was for HPV types 51 and 56, and the lowest for the HPV types 31, 33. The prevalence of HPV in this study was similar to surrounding countries and even some advanced countries, such as the United States and China, and was much less frequent in some of the surrounding countries, such as Turkey(Damin et al., 2007). It seems that HPV prevalence depended to geographical variation, cultural, religious and economic differences in different communities as well as insufficient numbers of samples examined in studies. Therefore, colorectal cancer is one of the most common malignancies around the world and the presence of a viral infection such as HPV can have a potential role in it.

In conclusion, the results provide quantitative evidence for an association between HPV infection and colorectal cancer risk. This is the first report showing the prevalence of HPV infections in Iranian (Kerman Province) colorectal tumors. Recent studies have shown that 15% of the existing cancers are due to infection with oncogenic viruses, which presence of papillomavirus is commonly found in human tumors, as it is an etiologic factor of cervical cancer. The presence of DNA in the colorectal cancer, as one of the most common cancers in developed countries, suggests its potential as a carcinogenic factor in this cancer. There are several risk factors for developing a tumor, one of which is the infection with oncogenic viruses such as HPV. Further studies are needed to investigate the relationship between HPV and colorectal cancer with more sample size and high accuracy diagnostic methods.

Conflicts of interest

None.

Acknowledgments

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